(19) World Intellectual Property Organization International Bureau



(43) International Publication Date 15 November 2001 (15.11.2001)

PCT

(10) International Publication Number WO 01/86288 A2

(51) International Patent Classification⁷: G01N 33/48

(21) International Application Number: PCT/EP01/05054

(22) International Filing Date: 4 May 2001 (04.05.2001)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data: MI2000A001002 8 May 2000 (08.05.2000) IT

(71) Applicant (for all designated States except US): MACROCHIP S.R.L. [IT/IT]; Via Padriciano, 99, 1-34100 Trieste (IT).

(72) Inventor; and

(75) Inventor/Applicant (for US only): STANTA, Giorgio [IT/IT]; Località Aurisiana S. Croce, 9/i, I-34011 Duino Aurisiana (IT).

(74) Agent: MODIANO, Guido; Modiano & Associati, Via Mcravigli, 16, I-20123 Milano (IT).

(81) Designated States (national): AE. AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DZ, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, TZ, UA, UG, US, UZ, VN, YU, ZA, ZW.

(84) Designated States (regional): ARIPO patent (GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE, TR), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).

Published:

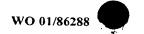
 without international search report and to be republished upon receipt of that report

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

₹ œ

(54) Title: METHOD AND APPARATUS FOR EARLY DIAGNOSIS OF BLADDER TUMOR IN URINE SAMPLES

(57) Abstract: A method for early diagnosis of bladder tumor in a urine sample, which comprises a step of amplification of the RNA extracted from cells present in the urine by using a marker for the messenger RNA of the catalytic component of telomerase (hTRT), a marker for β-actin to demonstrate RNA accessibility and as standard for quantitative estimation, in combination with at least one additional marker chosen from the group that comprises: a marker for a protein of the cytokeratin family, a lymphocyte marker which is suitable to detect inflammatory cells associated with neoplastic infiltration, and a final step of detecting the amplified material.



METHOD AND APPARATUS FOR EARLY DIAGNOSIS OF BLADDER TUMOR IN URINE SAMPLES

Technical Field

5

10

15

20

25

30

The present invention relates to a method and an apparatus for early diagnosis of bladder tumor in urine samples.

In particular, the present invention relates to a method and to the corresponding diagnostic apparatus for detecting, from the onset, even initial tumor forms affecting the mucous membrane of the bladder without resorting to the use of more or less invasive methods.

The method according to the invention is particularly suitable for detecting transitional carcinomas of the bladder, which make up almost all of the tumors affecting this region.

Background art

It is known that transitional bladder cell carcinoma (TCC) is one of the most widespread tumor in Western societies. At the time of diagnosis, 20% of TCCs are found to be of the invasive type, while 80% are superficial. 20% of the latter can evolve into an invasive form with infiltration of the muscular tunic of the bladder wall.

Due to the frequent relapses of these forms of tumor, periodic monitoring of patients, including asymptomatic ones, is indispensable.

However, it has been found that cystoscopy, which is currently the most widely used monitoring method because it provides the most reliable results, cannot be considered a method suitable for the screening of a large number of patients due to invasive nature of the procedure, which is particularly unpleasant in male patients.

An alternative non-invasive diagnostic method is cytological examination of urine. This method has the disadvantage of low sensitivity, which allows to recognize the neoplastic lesion only in 40-50% of investigated cases.

Transitional bladder tumor usually affects the elderly population, which

10

15

20

25

30



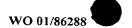
is increasing considerably in developed societies. This leads to the expectation of an increase in the number of requests for diagnosis of these tumors in the future. Since the analyses to which patients are subjected for accurate diagnosis are mostly invasive, an alternative, noninvasive, high-sensitivity and high-specificity method would be very important. Cytology, which is a noninvasive method, has low sensitivity and is also affected by the variability of the interpretation of the people who perform the test. Commercially available alternative methods are based on determining the various tumor-specific antigens (BTA, bladder tumor associated analytes (Bard Diagnostics, Redmond, Washington), NMP 22 nuclear matrix protein [Matritech, Newton, Massachusetts]). Although these methods are more sensitive than cytological examination, their stated clinical usefulness is compromised by the extreme variability due to the inflammatory processes, which can interfere with their sensitivity and specificity.

It is known that molecular techniques, based on DNA and RNA analysis, are extremely sensitive and specific and are therefore suggested increasingly also in clinical diagnostics.

It is well-known that in bladder tumor the pattern of genetic alterations, which cause the development of neoplastic cells, is highly complex, making analysis particularly troublesome. One of the first mutations studied at the level of tumor cells (ras oncogene mutation) has been found in bladder tumor cell lines and rarely in primitive bladder tumor.

Sidransky et al. (Science (1991) 252, 252-253) found point mutations of the oncosuppressor gene p53 in 61% of bladder tumor cases. Due to this relatively low frequency of mutations and to the large number of variations in said mutations, this investigation method cannot be used in clinical diagnostics.

Cytogenetic methods have revealed some chromosomal deletions at 9q, 11p and 17p, where oncosuppressor genes may be located. In order to identify loss of heterozygosity and gene instability, microsatellite markers



10

15

20

25

30

have an important application. These genetic alterations at the microsatellite level, however, are occasional, and in a significant number of tumors might not be revealed despite using a large number of microsatellite markers. Mao et al. (Science (1996) 271, 659-662) used a group of 13 tri- and tetranucleotide markers (it has been found that the longest repeats are those subjected to deletions or expansion). 95% of tumor cases (19 out of 20 cases) had an identical clonal alteration in the urine sample and in tumor biopsies.

Although the method has been found to be sensitive, it is not suitable for screening because it is extremely labor-intensive (large number of individuals to be analyzed).

The following diagnostic kits are currently commercially available:

- -- BTA (bladder tumor associated analytes), which has 74% sensitivity and 73% specificity. False positives have been found in patients without a bladder tumor but with considerable hematuria (6 out of 7 cases).
- -- NMP22 (nuclear matrix protein), which has 53% sensitivity and 60% specificity.
- -- FDP (fibrin and fibrinogen degradation products), which has 52% sensitivity and 91% specificity (The Journal of Urology (1999) 161, 388-394).

Currently, the need is felt to have a new method, with better characteristics, for the diagnosis and for following the evolution of bladder tumor, which is highly sensitive and specific, does not require harmful reagents and can be used directly on the urine of patients.

Determination of telomerase activity in cells obtained by bladder washing has recently been proposed (Clin. Canc. Research. (1998) 4, 535-538). Telomeres are the terminal portions of eukaryotic chromosomes and are meant to protect and stabilize chromosomes. Since DNA polymerase is unable to synthesize them during replication of somatic cells, telomeres gradually shorten. When the telomeres reach a limit length, chromosomal

10

15

20

25



instability leads to cell death (apoptosis). In immortalization of somatic cells and in malignant transformation, the telomeres stabilize and telomerase enzyme activity appears.

Telomerase is the enzyme responsible for telomere synthesis. It is constituted by three components (Current opinion in Genetics and Development (1999) 9, 97-103):

- 1) the RNA (hTR) component, which acts as a template for telomere synthesis (short repetitive sequences);
 - 2) the catalytic component hTRT;
 - 3) the telomerase-associated protein (TEP1).

The presence of the two components hTR and hTRT is sufficient to reconstruct telomerase activity in vitro.

Telomerase activity (addition of telomere repeats at the end of chromosomes, which occurs by using as template its RNA component [hTR]) is measured with the TRAP assay (telomeric repeat amplification protocol). Boehringer Mannheim (Roche Biochemicals) has developed a nonradioactive TRAP analysis system based on a method which uses an ELISA-type microplate. Since in excessively concentrated protein lysates the presence of Taq polymerase inhibitors may mask telomerase activity (false negatives), the TRAP assay must be performed by using various dilutions of the same sample. Furthermore, the presence of protease, RNase and the pH variations to which cells are exposed in urine can compromise the efficiency of the assay (false negatives).

Lee et al. (Clin. Can. Res. (1998) 4, 535-538) have demonstrated telomerase activity (TRAP assay) in 96% of tumor cases by performing analysis on tumor tissue and in bladder washes. Specificity is 96%. Heine (Journal of Pathology (1998) 184, 71-76) et al. have shown, with TRAP analysis, telomerase activity in 95% (19 out of 20) of bladder tumor tissues and in 70% (14 out of 20) of bladder washes, while activity was absent in urine sediment of bladder tumor patients. Rahat et al. (Cancer (1999) 85,

30

WO 01/86288

5

10

15

20

25

919-924) have demonstrated telomerase activity with TRAP analysis in 81% (17 out of 20) of urine samples of bladder tumor patients, with a specificity of 76%.

It has been demonstrated that telomerase activity is proportional to the quantity of messenger RNA of the protein component of telomerase (hTRT) (FEBS Letter (1999) 460, 285-288). Ito et al. (Clin. Can. Res. (1998) 4, 2870, 2810) have demonstrated that hTRT mRNA, the catalytic part of telomerase, is expressed in urine sediment in 76% (23 out of 33) of bladder tumor cases. The specificity of the method is 96%. Ito et al. (Clin. Can. Res. (1998) 4, 1603-1608) have provided studies on the distribution of the expression of all three components of telomerase, i.e., hTRT, hTR and TEP1, in bladder tumor tissues. hTRT is expressed in 90% of tumor tissues (30 out of 33) and in 20% of neighboring tissues. On the contrary, hTR and TEP1 are expressed both in tumor tissues and in normal tissues. Scientific literature has found no significant correlation between expression of hTRT and clinical-pathological characteristics of the tumors. Suzuki et al. (The Journal of Urology (1999) 162, 2217-2220) have demonstrated that hTRT is expressed in 100% of bladder tumor tissues (27 cases) and in none of the normal tissues that surround the tumor, while the TEP1 protein is expressed equally in tumor tissues and in normal tissues.

Disclosure of the Invention

A noninvasive method for early diagnosis of bladder tumor has now been found which is based on the simultaneous determination of the expression of the catalytic part of telomerase (hTRT) at the level of messenger RNA and of at least one additional selected molecular marker.

The diagnostic method according to the invention is based on the simultaneous determination of the messenger RNA of the protein component of telomerase, which is a marker for cell immortalization and therefore for the presence of cells in vivo which have undergone a malignant transformation, and of another selected marker.

30

10

15

20

25



In particular, the diagnostic method according to the invention is based on highlighting both the expression of the telomerase enzyme by quantization of the messenger RNA of the catalytic part (hTRT marker) in urine and the expression of at least one additional molecular marker, and on subsequent quantization thereof and analysis of the results obtained.

The method based on the interpretation of the response of two or more molecular markers increases sensitivity and specificity up to 100% of both. In this manner, the method according to the invention is suitable for screening and monitoring of patients undergoing treatment.

In accordance with a first aspect of the present invention, a diagnostic method for bladder tumor is provided which comprises a step of amplification of the RNA extracted from the cells present in a urine sample by using:

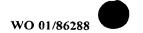
- -- an hTRT marker;
- -- a marker, preferably β-actin, as demonstration of RNA accessibility and as standard for quantitative estimation,

in association with at least one other marker chosen from the group that comprises:

- -- a marker for one of the proteins of the cytokeratin family;
- -- a marker for lymphocytes which is suitable to detect inflammatory cells associated with neoplastic infiltration.

In particular, the present invention provides a method for early diagnosis of bladder tumor in a urine sample which comprises:

- a) optionally, a preliminary step of extracting the total RNA from the cells that are present in the urine;
 - b) a step of amplification of the extracted RNA by using:
- -- a marker for the messenger RNA of the catalytic component of telomerase (hTRT);
 - -- a marker for β-actin
- in combination with at least one additional marker chosen from the group



that consists of:

5

15

20

25

- -- a marker for a protein of the cytokeratin family;
- -- a lymphocyte marker which is suitable to detect inflammatory cells associated with neoplastic infiltration;
 - and, advantageously, a thermocycler or real-time PCR
 - c) a step of detecting the amplified material.

One of the markers used in the invention belongs to the cytokeratin family, which is composed of 20 polypeptides which are part of the intermediate filaments of epithelial cells. Cytokeratins are expressed in various combinations according to the type of epithelial cell and to the degree of differentiation. Among cytokeratins, the use of cytokeratin 20 (CK20) is particularly suitable in the invention.

Furthermore, it has been found that among markers for lymphocytes, CD4 is particularly suitable to detect the presence of helper lymphocytes associated with neoplastic infiltration.

In particular, determination of the RNA of hTRT in combination with three other markers, such as cytokeratin 20, CD4 and \(\beta\)-actin, and subsequent coordinated interpretation of the results provide a means for a sensitive and specific diagnosis of bladder tumor directly in urine.

The step for amplification of extracted RNA according to the method of the invention advantageously comprises two steps:

- i) a reverse transcription (RT) step, performed by providing a specific antisense primer or primers and
- ii) an amplification step (PCR), using the respective specific sense primers, which are advantageously biotinylated in order to facilitate the subsequent detection step.

In accordance with one embodiment, the amplification step is performed with a thermocycler or real-time PCR.

According to another embodiment of the invention, the amplification step is performed in the presence of ddUTP-biotinylate.

10

15

20

25

30



The subsequent revelation step comprises:

-- hybridization of the products amplified in step b) in a first well which contains a probe for the amplified hTRT marker and at least one well which contains a probe for a marker for a protein of the cytokeratin family, a marker for a lymphocyte suitable to detect inflammatory cells associated with neoplastic infiltration, and β-actin. Alternatively, the biotinylated amplified material is bound to the plate with streptavidin and the amplification products are hybridized with the probe, preferably marked with DIG.

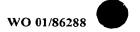
-- detection of the bound fragment (recognition that hybridization has occurred) with the probe used, which is preferably performed by using streptavidin, which recognizes biotin and with which alkaline phosphatase or peroxidase is advantageously bound.

In accordance with another embodiment, the amplified material, hybridized with the probe marked with DIG, is detected with anti-DIG with which alkaline phophatase or peroxidase is advantageously bound. Instrumental detection, in both cases (for example with an ELISA reader), is preferably performed with the addition of a colorimetric substrate or by chemiluminescence.

As an alternative to alkaline phosphatase or peroxidase, streptavidin is marked with molecules for fluorescence emission.

In the case of use of real-time PCR, which is capable of performing amplification in real time, the detection system used can be an intercalating staining solution, such as Syber green or specific probes marked with molecules for fluorescence emission.

The diagnostic method according to the invention allows to identify neoplastic cells of epithelial origin and CD4+ lymphocytes. In particular, the combination of markers according to the invention allows to obtain detailed information regarding the desquamation of neoplastic uroepithelial cells and the infiltration of the tumor on the part of CD4+ lymphocytes. The



10

15

20

25

method according to the invention allows to analyze the cells that are present in the urine even if they are not perfectly preserved, differently from the TRAP method, which leads to a false negative if the proteins are degraded.

The method according to the invention also avoids the need to perform bladder washing, since the urine of the patient can be used directly.

This last advantage is very important, since bladder washing is in any case a diagnostic intervention performed on the patient, with additional costs and discomfort for said patient.

The method according to the invention provides a particularly accurate indication of the existence of a malignant tumor form in progress, located at the bladder, and also provides considerable advantages with respect to the other methods used so far.

According to another aspect of the invention, an apparatus for the diagnosis or for monitoring bladder tumor is provided which comprises:

- -- a marker for the messenger RNA of the catalytic component of telomerase (hTRT), a marker for β-actin to demonstrate RNA accessibility and as standard for quantitative estimation, and at least one additional marker chosen from the group that comprises:
 - -- a marker for a protein of the cytokeratin family;
- -- a marker for a lymphocyte suitable to detect inflammatory cells associated with neoplastic infiltration.

In accordance to a preferred embodiment, said marker for a lymphocyte suitable to detect inflammatory cells associated with neoplastic infiltration is CD4, and said marker for a protein of the cytokeratin family is cytokeratin 20 or CK20.

Advantageously, the apparatus according to the invention furthermore comprises a plurality of ELISA plates and conveniently one or more of the above described reagents, markers, and probes.

The following examples are provided merely as a non-limitative



illustration of the present invention.

Example 1

5

10

15

20

25

30

A) Urine collection and storage

A human urine sample was processed according to the following procedure directly after collection:

- -- collection of urine sediment by passing the urine to be tested through an appropriate filter, with application of vacuum (Talent srl);
- -- the filter containing the cells was immersed in a lysis solution ("Total Quick RNA" kit by Talent), centrifuged for 1 minutes, and left immersed for 10 minutes.

The solution, after removing the filter, was stored at -80 °C.

B) Extraction of total RNA.

Extraction was performed (by means of the "Total Quick RNA" method by Talent, Trieste, Italy) by using the specific column. The RNA bound selectively with the resin, which was subsequently washed to eliminate traces of protein and DNA. The RNA was then eluted with warm water.

C) Diagnostic investigation according to the invention.

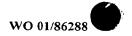
The method was conducted on an ELISA-type microplate.

I) Step of amplification of extracted RNA. This step was performed by using four specific markers and a thermocycler.

Amplification occurred in two steps:

- 1) reverse transcription by using a specific antisense primer
- 2) amplification by using the respective biotinylated specific sense primers (for the subsequent revelation procedure).
 - II) a detection step.

The amplification products were hybridized in four different wells. Each well contained one of the four specific probes: telomerase, CK20, CD4 and ß-actin. For each marker, positive and negative control amplification was performed, with subsequent hybridization on a microplate. The probe was complementary to the fragment amplified with the biotinylated primer.



10

20

25

Detection of the bound fragment occurred by means of streptavidin (which recognizes biotin), to which alkaline phosphatase was bound. After adding the colorimetric substrate (4-nitrophenyl phosphate) or chemiluminescence substrate, reading occurred with the appropriate instrument (for example with an ELISA reader).

The streptavidin was alternatively bound with a fluorochrome which was detected by virtue of an appropriate system (for example with an ELISA reader).

The cutoff levels of the CD4 and CK20 markers were chosen from the first series of experiments, using the combination of three markers (hTRT, CK20 and CD4) in a small number of cases with neoplasms and phlogosis. The cases that expressed even a minimal quantity of hTRT were considered positive. All the markers were compared with the values of \(\beta\)-actin.

Interpretation of results:

The cases in which two or three markers were positive (in the case of CK20 and CD4, above the preset threshold) were considered positive (+) for tumor. Using this approach, the tumors (27 out of 31) that had already been diagnosed at the clinical level and the negative cases that also reflected the clinical diagnosis of cystitis were defined.

The cases in which only one marker of the three proposed ones (hTRT, CK20 and CD4) was positive were defined as inconclusive (10 cases). Of these, 40% (4 cases) were found to be tumors (comparing the results with clinical results) and 60% were clinically negative.

Since the tumor cases defined as inconclusive are proposed for subsequent retesting, the method according to the invention has 100% sensitivity (27 tumor cases diagnosed with our method + 4 cases defined as inconclusive). The specificity of the method was 45% (5 cases of cystitis out of 11), but if the inconclusive cases are considered, specificity is 100%. In other words, a tumor was never defined as negative and a cystitis was never considered positive.

30



Interpretation of the results allowed to define 3 groups:

- 1) Positive for at least two markers -- tumor (all cases were found to be real tumors)
- 2) Negative for all three markers -- absence of tumor (none of the cases defined as negative had neoplasms)
 - 3) Inconclusive cases because only one of the markers is positive: investigation to be repeated or confirmed with other methods (of the 10 cases defined as inconclusive, 4 were then found to be tumor).

Considering the sensitivity and specificity values expressed by the individual markers, the following values were found:

- 1) For hTRT: 94% sensitivity, 82% specificity
- 2) For CK20: 74% sensitivity, 64% specificity
- 3) For CD4: 52% sensitivity and 100% specificity

The following Table 1 is an interpretation of the quantitative results on 42 patients (31 with tumor) expressed as sensitivity and specificity. The specificity and sensitivity of our method are compared with other noninvasive methods.

TABLE 1

Analysis	Sensitivity	Specificity
hTRT marker	94%	82%
CK20 marker	74%	64%
CD4 marker	52%	100%
Markers: hTRT + CK20 + CD4 + inconclusive	100%	100%
cases (after second analysis performed on		
inconclusive cases by taking a new sample of		
the same patient)		
TRAP	70%	99%
BTA	74%	73%
NMP22	53%	62%

WO 01/86288

Cytology	44%	95%
FDP	52%	95%

13

The following Table 2 lists the results found in 42 cases with neoplasms and phlogosis. The results show that the cases found positive simultaneously for the hTRT marker, the cytokeratin marker and the lymphocyte marker according to the present invention led to a diagnosis of bladder tumor which was confirmed clinically, indicating a high reliability of the diagnostic results found with the method according to the present invention.

TABLE 2

Cases	hTRT	CD4	CK20	Diagnosis with	Clinical
				our method	diagnosis
1	+	-	+	Tumor	Tumor
2	_	-	-	Negative	Cystitis
3	+	+	+	Tumor	Tumor
4	_		+	Inconclusive	Cystitis
5	_	-	+	Inconclusive	Cystitis
6	-		-	Negative	Cystitis
7	+	_	<u>-</u>	Inconclusive	Tumor
8	+	+	-	Tumor	Tumor
9	+	-	+	Tumor	Tumor
10	_	-	-	Negative	Cystitis
11	+		+	Tumor	Tumor
12	+	-	+	Tumor	Tumor
13	+	-	+	Tumor	Tumor
14	+	+	-	Tumor	Tumor
15	-	_	_	Negative	Cystitis
16	-	_	+	Inconclusive	Cystitis
17	-	-	+	Inconclusive	Cystitis



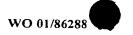
	14					
18	+		+	Tumor	Tumor	
19	+		-	Inconclusive	Tumor	
20			+	Inconclusive	Tumor	
21	· +	-	+	Tumor	Tumor	
22	+	_	+	Tumor	Tumor	
23	+	_	_	Inconclusive	Cystitis	
24	+	-	+	Tumor	Tumor	
25	+	+	+	Tumor	Tumor	
26	+	+	+	Tumor	Tumor	
27	+	-	+	Tumor	Tumor	
28	+	+	-	Tumor	Tumor	
29	+	+	-	Tumor	Tumor	
30	+	+		·Tumor	Tumor	
31	.+	+	+	Tumor	Tumor	
32	+	+	+	Tumor	Tumor	
33	+	+	+	Tumor	Tumor	
34	+	+	+	Tumor	Tumor	
35	+	-		Inconclusive	Cystitis	
36	+	+		Tumor	Tumor	
37	-	+	+	Tumor	Tumor	
38	-	+	+	Tumor	Tumor	
39	+	<u>.</u>	+	Tumor	Tumor	
40	+	-	+	Tumor	Tumor	
41	<u>-</u>	-	_	Cystitis	Cystitis	
42	-	+	-	Inconclusive	Tumor	

Example 2

Reagents and instruments used to provide an embodiment of the method according to the invention:

For RNA extraction

SDOCID: <WO 018628842 I



- -- absolute ethanol
- -- 75% ethanol
- -- microcentrifuge
- -- sterile tips

5 For RT-PCR

10

-- thermocycler

For detection

-- ELISA reader

Content of an embodiment of a diagnostic kit:

- 1) Accessories for collecting urine sediment
 - 2) Kit for RNA extraction
 - 3) Kit for RT-PCR
 - 4) Detection kit

Kit for RNA extraction

- 15 TQ-RNA Lysing solution
 - TQ-RNA Binding resin
 - TQ-RNA Washing solution A
 - TQ-RNA Washing solution B
 - TQ-RNA Conditioning solution
- 20 TQ-RNA Spin columns
 - 2 ml collection tubes
 - 1.5 collection tubes

Accessories for collecting urine sediment

Urine collection filters

25 Vacuum manifold

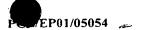
Kit for RT-PCR

Primer up (biotinylated)

Primer down

Reverse transcriptase

30 5x buffer for reverse transcriptase



10x buffer for PCR

Taq polymerase

Kit for detection

- -- ELISA plates (4 plates, each containing one of the four specific probes:
- telomerase, CK20, CD4 and β-actin)
 - -- Reagent 1 (blocking reagent)
 - -- Reagent 2 (hybridization solution)
 - -- Reagent 3A (washing solution)
 - -- Reagent 3B (washing solution)
- 10 -- Streptavidin, to which alkaline phosphatase is bound
 - -- Reagent 4
 - -- Reagent 5 (washing solution)
 - -- Reagent 6 (substrate solution)
 - -- Colorimetric substrate (4-nitrophenyl phosphate)
- -- Reagent 7

Urine collection and storage

The urine samples must be processed as quickly as possible:

- 4) urine sediment was collected by vacuum and appropriate filters (supplied by Talent).
- 5) the filter containing the cells was immersed in the lysing solution ("Total Quick RNA" kit by Talent), centrifuged for 1 minute, and left immersed for 10 minutes.
 - 6) the solution, after removing the filter, was stored at -80°C.

Extraction of total RNA with the "Total Quick RNA" method by Talent:

Extraction was performed with the "Total Quick RNA" method by Talent, which is a quick method (20 minutes) based on column centrifugation. The RNA was specifically bound to the resin, which was then washed to eliminate traces of protein and DNA, and then eluted with warm water.

30 <u>RT-PCR</u>

RNA concentration was measured at 260 nm absorbance. Absorbance equal to 1 unit corresponds to 40 µg of RNA per ml.

The step for amplification of the extracted RNA by using four specific markers and a thermocycler occurred in two steps:

- 1) reverse transcription by using a specific antisense primer (RT)
 - 2) amplification (PCR) by using the respective specific sense primers which are biotinylated (for subsequent revelation of bound fragments).

RT

Preparation of mix:

10	5x buffer	$2\mu l$
	Primer down (30 pmol/µl)	0.5 µl
	Mixture of NTPs	1 μ1
	AMV	$0.1~\mu l$
	RNase	0.1
15	H_2O	5.3 µl
	RNA (500 ng/µl)	1 μl
	Total volume	10 μl

Incubation at 42°C was performed for 1 hour.

PCR

25

20 Preparing the mix:

10x buffer	5 µl
Primer up (biotinylated)	0.5 µl
Taq polymerase	0.25 µl
H_2O	34.25 µl
Total volume	40 ul

The mix was added in the respective tubes of the RT and amplified by performing the following cycles:

95 °C for 3 minutes

5 cycles of:

30 95 °C for 1 minute



- 55 °C for 1 minute
- 72 °C for 1 minute
- 40 cycles of:
- 95 °C for 30 seconds
- 55 °C for 30 seconds
- 72 °C for 30 seconds

Detection

10

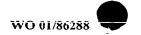
15

The amplification products were adsorbed on four different wells (for example, from a total of 50 μ l, 10 μ l per well were placed (each well contained one of the four specific probes: telomerase, CK20, CD4 and β -actin). The probe was complementary to the fragment amplified with the biotinylated primer.

Detection of the bound fragment occurred by means of streptavidin (which recognizes biotin), to which the alkaline phosphatase was bound. After adding the colorimetric substrate (4-nitrophenyl phosphate), reading was performed with an ELISA reader.

The analysis procedure entailed performing the following steps:

- 1) 200 µl of Reagent 1 were added in the wells of the ELISA and incubation was performed at ambient temperature for one hour under agitation.
- 20 2) the amplified material was denatured at 95 °C for 10 minutes, placing it immediately in ice.
 - 3) the wells were emptied. 90 µl of Reagent 2 and 10 µl of each amplified product were added in the respective wells, and incubation was performed at 50 °C for two hours.
- 25 4) The wells were emptied and washed 3 times with 200 μl of Reagent 3A heated at 50 °C.
 - 5) The wells were incubated with Reagent 3A at 50 °C for 15 minutes.
 - 6) Emptying and washing were performed 3 times with 200 μ l of Reagent 3B heated to 50 °C.
- 30 7) For each well, 100 μl of streptavidin, conjugated with alkaline



phosphatase, diluted at 1:5000 in Reagent 4, were added.

- 8) Incubation was performed at 50 °C for 1 hour.
- 9) The wells were emptied and washed 3 times with 200 μ l of Reagent 5 at ambient temperature.
- 5 10) The wells were incubated with Reagent 5 for 5 minutes at ambient temperature.
 - 11) Washing was performed 3 times with 200 μ l of Reagent 5 at ambient temperature.
 - 12) 100 µl of pNPP, 10 mg/ml, dissolved in solution 6, were added.
- 13) Incubation was performed for 30 minutes at ambient temperature in darkness.
 - 14) The reaction was blocked with 100 μ l of Reagent 7.
 - 15) The results were read at 405 nm.

The disclosures in Italian Patent Application No. MI2000A001002 from which this application claims priority are incorporated herein by reference.

10

15

20

25

30



CLAIMS

20

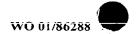
- 1. A method for early diagnosis of bladder tumor in a urine sample, characterized in that it comprises the determination, on the RNA extracted from the cells present in the urine, of:
- -- a marker for the messenger RNA of the catalytic component of telomerase (hTRT) and a marker for β-actin, to demonstrate RNA accessibility and as standard for quantitative estimation,

in association with at least one additional molecular marker chosen from the group that comprises:

- -- a marker for a protein of the cytokeratin family;
- -- a lymphocyte marker which is suitable to detect inflammatory cells associated with neoplastic infiltration.
- 2. A method for early diagnosis of bladder tumor in a urine sample, comprising:
- a) a step of amplification of the RNA extracted from cells present in the urine by using:
- -- a marker for the messenger RNA of the catalytic component of telomerase (hTRT), a marker for β-actin to demonstrate RNA accessibility and as standard for quantitative estimation,

in combination with at least one additional marker chosen from the group that comprises:

- -- a marker for a protein of the cytokeratin family;
- -- a lymphocyte marker which is suitable to detect inflammatory cells associated with neoplastic infiltration;
 - b) a step of detecting the amplified material.
- 3. The method according to claim 2, characterized in that said protein marker of the cytokeratin family is cytokeratin 20 (CK20).
- 4. The method according to claim 2 or 3, characterized in that said lymphocyte marker suitable to detect inflammatory cells associated with the neoplastic infiltration is CD4.



10

15

20

- 5. The method according to claim 2, characterized in that said amplification comprises:
 - i) reverse transcription by using a specific antisense primer;
 - ii) amplification by using specific sense primers.
- 6. The method according to claim 2, characterized in that said specific sense primers of the amplification step ii) are preferably biotinylated in order to facilitate the subsequent detection step.
- 7. The method according to claim 2, characterized in that said detection step comprises hybridization of the amplified products with probes which are specific for the markers used in said step b) and the subsequent recognition that hybridization has occurred with said probe preferably by means of streptavidin.
- 8. The method according to claim 7, characterized in that said streptavidin is conjugated with alkaline phosphatase or with peroxidase for colorimetric or chemiluminescence detection.
- 9. The method according to claim 7, characterized in that said streptavidin is conjugated with fluorescein for fluorescence detection.
- 10. The method according to claim 2, characterized in that said amplification products of step b) are adsorbed on a first well which contains a probe which is immobilized for hTRT and on at least one additional well which contains a probe for the marker used in said step b).
- 11. The method according to any one of the preceding claims 1 to 10, characterized in that it comprises a preliminary step of extracting the total RNA from the cells that are present in the urine.
- 25 12. An apparatus for diagnosis or for monitoring bladder tumor, characterized in that it comprises:
 - -- a messenger RNA marker for the catalytic component of telomerase (hTRT), a \(\beta\)-actin marker to demonstrate RNA accessibility and as a standard for quantitative estimation, and at least one additional marker chosen from the group that comprises:

30

15

20



- -- a protein marker of the cytokeratin family;
- -- a lymphocyte marker suitable to detect inflammatory cells associated with neoplastic infiltration.
- 13. The apparatus according to claim 12, characterized in that said lymphocyte marker suitable to detect inflammatory cells associated with neoplastic infiltration is CD4.
- 14. The apparatus according to claim 12 or 13, characterized in that said protein marker of the cytokeratin family is cytokeratin 20 or CK20.
- 15. The apparatus according to any one of claims 12 to 14, characterized in that it furthermore comprises a plurality of ELISA plates.
- 16. The apparatus according to claim 15, characterized in that it comprises 4 ELISA plates, each of which comprises one of the four specific probes: telomerase, CK20, CD4, and β-actin.
- 17. The apparatus according to any one of claims 12 to 16, characterized in that it furthermore comprises streptavidin to which alkaline phosphatase or peroxidase is linked.
- 18. The apparatus according to any one of claims 12 to 17, characterized in that it furthermore comprises one or more washing solutions.
- 19. The apparatus according to any one of claims 11 to 18, characterized in that it furthermore comprises a solution for hybridization and a colorimetric substrate.
- 20. The apparatus according to any one of claims 12 to 16 and 18-19, characterized in that it furthermore comprises streptavidin conjugated with fluorescein for fluorescence detection.

THIS PAGE BLANK (USPTO)

(19) World Intellectual Property Organization International Bureau





(43) International Publication Date 15 November 2001 (15.11.2001)

PCT

(10) International Publication Number WO 01/86288 A3

- (51) International Patent Classification⁷: G01N 33/574, C12Q 1/68
- (21) International Application Number: PCT/EP01/05054
- (22) International Filing Date: 4 May 2001 (04.05.2001)
- (25) Filing Language:

English

(26) Publication Language:

English

(30) Priority Data: M12000A001002

8 May 2000 (08.05,2000) IT

- (71) Applicant (for all designated States except US):
 MACROCHIP S.R.L. [IT/IT]; Via Padriciano, 99,
 1-34100 Trieste (IT).
- (72) Inventor; and
- (75) Inventor/Applicant (for US only): STANTA, Giorgio [IT/IT]: Località Aurisiana S. Croce. 9/i. 1-34011 Duino Aurisiana (IT).
- (74) Agent: MODIANO, Guido: Modiano & Associati, Via Meravigli, 16, I-20123 Milano (IT).

- (81) Designated States (national): AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DZ, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, TZ, UA, UG, US, UZ, VN, YU, ZA, ZW.
- (84) Designated States (regional): ARIPO patent (GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE, TR), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).

Published:

- with international search report
- before the expiration of the time limit for amending the claims and to be republished in the event of receipt of amendments
- (88) Date of publication of the international search report: 16 May 2002

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

(54) Title: EARLY DIAGNOSIS OF BLADDER TUMOR IN URINE SAMPLES

(57) Abstract: A method for early diagnosis of bladder tumor in a urine sample, which comprises a step of amplification of the RNA extracted from cells present in the urine by using a marker for the messenger RNA of the catalytic component of telomerase (hTRT), a marker for β -actin to demonstrate RNA accessibility and as standard for quantitative estimation, in combination with at least one additional marker chosen from the group that comprises: a marker for a protein of the cytokeratin family, a lymphocyte marker which is suitable to detect inflammatory cells associated with neoplastic infiltration, and a final step of detecting the amplified material.

A. CLASSIFICATION OF SUBJECT MATTER IPC 7 G01N33/574 C12Q1/68

C. DOCUMENTS CONSIDERED TO BE RELEVANT

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols) IPC 7 G01N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

EPO-Internal, BIOSIS, CHEM ABS Data, MEDLINE, WPI Data, PAJ

Category °	Citation of document, with indication, where appropriate, of	Relevant to claim No.	
X	EP 0 926 245 A (ROCHE DIAGNOS 30 June 1999 (1999-06-30) the whole document		
x	DE KOK, JACQUES B. (1) ET AL: quantification of human telom transcriptase mRNA in tumors tissues." CLINICAL CHEMISTRY, (MARCH, 2 NO. 3, PP. 313-318., XP002176598 abstract	erase reverse and healthy	1,2,12
		-/	
χ Furt	her documents are listed in the continuation of box C.	χ Patent family members are listed	in annex.
Special ca 'A' docum consid 'E' earlier filing o 'L' docum which citatio 'O' docum other 'P' docum	ategories of cited documents : ent defining the general state of the art which is not dered to be of particular relevance document but published on or after the international	Table of the cannot be considered to the cannot be considered to volve an inventive step when the cannot be considered novel or cannot in volve an inventive step when the document of particular relevance; the cannot be considered novel or cannot inventive step when the document of particular relevance; the cannot be considered to involve an in document is combined with one or ments, such combination being obvious the art. '&' document member of the same patent.	ernational filing date the application but eory underlying the claimed invention to considered to ocument is taken alone claimed invention eventive step when the ore other such docu- us to a person skilled
Special ca 'A' docume consid 'E' earlier filing of 'L' docume which citatio 'O' docume other 'P' docume later t	ategories of cited documents: ent defining the general state of the art which is not dered to be of particular relevance document but published on or after the international date ent which may throw doubts on priority claim(s) or is cited to establish the publication date of another in or other special reason (as specified) ent referring to an oral disclosure, use, exhibition or means ent published prior to the international tilting date but	'T' later document published after the inte or priority date and not in conflict with cited to understand the principle or the invention invention 'X' document of particular relevance; the cannot be considered novel or cannot involve an inventive step when the do 'Y' document of particular relevance; the cannot be considered to involve an indocument is combined with one or ments, such combination being obvious in the art.	ernational filing date the application but eory underlying the claimed invention t be considered to ocument is taken alone claimed invention inventive step when the ore other such docu- ius to a person skilled
Special car 'A' docume conside 'E' earlier filing of 'L' docume which citatio 'O' docume other 'P' docume later to the consider of the consideration of the c	ategories of cited documents: ent defining the general state of the art which is not dered to be of particular relevance document but published on or after the international date ent which may throw doubts on priority claim(s) or is cited to establish the publication date of another in or other special reason (as specified) ent referring to an oral disclosure, use, exhibition or means ent published prior to the international filling date but than the priority date claimed	'T' later document published after the integration or priority date and not in conflict with cited to understand the principle or the invention 'X' document of particular relevance; the cannot be considered novel or cannot involve an inventive step when the document of particular relevance; the cannot be considered to involve an indocument is combined with one or ments, such combination being obvious in the art. '&' document member of the same patent	ernational filing date the application but eory underlying the claimed invention t be considered to ocument is taken alone claimed invention inventive step when the ore other such docu- ius to a person skilled

INTERNATION SEARCH REPORT

PCT/EP 01/05054

<u></u>		PCT/EP 01/05054			
C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT					
Category *	Citation of document, with indication, where appropriate, of the relevant passages	Ficlevant to claim No.			
X	BIALKOWSKA-HOBRZANSKA, HANNA ET AL: "Comparison of human telomerase reverse transcriptase messenger RNA and telomerase activity as urine markers for diagnosis of	1,2,12			
	bladder carcinoma" MOL. DIAGN. (2000), 5(4), 267-277 , XP008000978 abstract				
X	MUELLER, MARKUS (1) ET AL: "Diagnosis of bladder cancer by detecting: Different telomerase subunits in urine." JOURNAL OF CLINICAL LIGAND ASSAY, (WINTER, 1999) VOL. 22, NO. 4, PP. 354-357., XP001029128 abstract	1,2,12			
A	EP 0 841 396 A (GERON CORP ;UNIV TECHNOLOGY CORP (US)) 13 May 1998 (1998-05-13) abstract	1,2,12			
A	US 5 863 726 A (HARLEY CALVIN BRUCE ET AL) 26 January 1999 (1999-01-26) abstract	1,2,12			
Ą	WO 97 35871 A (SINAI SCHOOL MEDICINE) 2 October 1997 (1997-10-02) abstract	1,2,12			
Α	EP 0 930 369 A (CHUGAÍ PHARMACEUTICAL CO LTD) 21 July 1999 (1999-07-21) abstract 	1,2,12			

ERNATIONAL SEARCH REPORT

ormation on patent family members

PC1/EP 01/05054

		_	PCI/EP	01/05054
Patent document cited in search report	Publication date		Patent family member(s)	Publication date
EP 0926245	A 30-06-1999	DE	19757300 A1	24-06-1999
		EP	0926245 A2	30-06-1999
		JP	11243995 A	14-09-1999
EP 0841396	A 13-05-1998	US	6093809 A	25-07-2000
2. 55.1255	. 10 00 1370	US	6261836 B1	17-07-2001
		ΑU	4803697 A	24-04-1998
		AU	734089 B2	07-06-2001
		AU	4807397 A	24-04-1998
		BR BR	9711844 A 9712254 A	18-01-2000 18-01-2000
		CH	689672 A5	13-08-1999
		CN	1291231 A	11-04-2001
		CN	1254376 A	24-05-2000
		DE	19743497 A1	20-08-1998
		DE	841396 T1	24-09-1998
		EP EP	08 4 1396 A1 0932686 A2	13-05-1998 04-08-1999
		EP	0954585 A2	10-11-1999
		FΙ	990655 A	24-03-1999
		FR	2757177 A1	19-06-1998
		GB	2317891 A ,B	08-04-1998
		GB JP	2321642 A ,B 11313689 A	05-08-1998 16-11-1999
		JP	10234384 A	08-09-1998
		JP	11253177 A	21-09-1999
		JP	2001523947 T	27-11-2001
		JP	2001510985 T	07-08-2001
		JP	2001081042 A	27-03-2001 31-05-1999
		NO WO	991588 A 9814592 A2	09-04-1998
-		WO	9814593 A2	09-04-1998
		ÜS	6309867 B1	30-10-2001
		US	6166178 A	26-12-2000
US 5863726 A	26-01-1999	US	5837453 A	17-11-1998
03 3803720 A	20-01-1999	US	5629154 A	13-05-1997
		ÜS	5989807 A	23-11-1999
		US	5830644 A	03-11-1998
		US	5645986 A	08-07-1997
		AU JP	6380896 A 11507839 T	15-05-1997 13-07-1999
		WO	9715687 A1	01-05-1997
		US	5891639 A	06-04-1999
		US	5804380 A	08-09-1998
		AT	193554 T	15-06-2000
		AU AU	682082 B2 1209095 A	18-09-1997 29-05-1995
		AU	6058298 A	04-06-1998
		CA	2173872 A1	18-05-1995
		DE	69424797 D1	06-07-2000
		DE	69424797 T2	28-12-2000
		DK EP	728207 T3 0728207 A1	02-10-2000 28-08-1996
		ES	2147602 T3	16-09-2000
		GR	3034249 T3	29-12-2000
		JP	11243998 A	14-09-1999
		JP	2875394 B2	31-03-1999
L				

INTERNATIONAL EARCH REPORT

formation on pater amily members

PCT/EP 01/050-4

Patent document cited in search report	1	Publication date		Patent family member(s)	Publication date
US 5863726	A	<u> </u>	JP	9502102 .T	04-03-1997 30-11-2000
			PT WO	728207 T 9513381 A1	18-05-1995
			US	5648215 A	15-07-1997
٠			US	5639613 A	17-06-1997
			US	5693474 A	02-12-1997
•			ΑU	1178195 A	29-05-1995
			ΑU	1330795 A	29-05-1995
		• .	WO	9513382 A1	18-05-1995
			US	5686306 A	11-11-1997
			WO	9513383 A1	18-05-1995
WO 9735871	A	02-10-1997	AU	2547697 A	17-10-1997
WO 3733371	,,	02 10 1557	JP	9262100 A	07-10-1997
•			WO	9735871 A1	02-10-1997
EP 0930369	 A	21-07-1999	AU	3276497 A	21-01-1998
			EP	0930369 A1	21-07-1999
			WO	9800563 A1	08-01-1998
			JP	10071000 A	17-03-1998

COTTON AND A STREET OF THE

THIS PAGE BLANK (USPTO)

This Page is Inserted by IFW Indexing and Scanning Operations and is not part of the Official Record

BEST AVAILABLE IMAGES

Defective images within this document are accurate representations of the original documents submitted by the applicant.

Defects in the images include but are not limited to the items checked:

BLACK BORDERS

IMAGE CUT OFF AT TOP, BOTTOM OR SIDES

FADED TEXT OR DRAWING

BLURRED OR ILLEGIBLE TEXT OR DRAWING

SKEWED/SLANTED IMAGES

COLOR OR BLACK AND WHITE PHOTOGRAPHS

GRAY SCALE DOCUMENTS

LINES OR MARKS ON ORIGINAL DOCUMENT

REFERENCE(S) OR EXHIBIT(S) SUBMITTED ARE POOR QUALITY

IMAGES ARE BEST AVAILABLE COPY.

☐ OTHER: ____

As rescanning these documents will not correct the image problems checked, please do not report these problems to the IFW Image Problem Mailbox.

THIS PAGE BLANK (USPTO)